Respiratory syncytial virus: recent progress towards the discovery of effective prophylactic and therapeutic agents

Nicholas A. Meanwell and Mark Krystal

Although respiratory syncytial virus (RSV) was discovered in 1955, the burden associated with this infectious agent on all population groups is only now beginning to be fully appreciated. The successful launch of the humanized monoclonal antibody Synagis (developed by MedImmune, Gaithersburg, MD, USA), as a prophylactic in September 1998 has helped to heighten awareness of the extent of mortality and morbidity associated with annual RSV epidemics. Small, drug-like molecules that would provide the clinician with effective and conveniently administered prophylactic and therapeutic agents for the prevention and treatment of RSV have not yet advanced into clinical studies. This review will summarize recent developments in the area of RSV drug discovery and development.

Respiratory syncytial virus (RSV) was originally identified in 1955 (Ref. 1) after an outbreak of infection in chimpanzees. This was quickly followed by the demonstration that this virus represented a new human pathogen, after being detected in lung secretions from individual infants presenting with pneumonia[2] and bronchiolitis[3]. The prevalence of RSV was subsequently revealed by serological studies, which indicated that 80% of children under four years of age possessed neutralizing antibodies to RSV in their serum[3]. Over the ensuing 20 years, a series of epidemiological studies has established the pattern of RSV epidemics and documented the importance of RSV as the leading cause of lower respiratory tract infection in infants and children[4,5]. More recent clinical studies have probed the role of RSV infection either as the cause of clinical symptoms or as an exacerbating agent in a variety of pathophysiological conditions. As a consequence, it is now becoming apparent that RSV is a greatly underestimated disease burden at all stages of life. Moreover, the current level of understanding of the biochemical pharmacology of RSV is considerably less than that of influenza, for which RSV infection is frequently mistaken[6]. In addition, animal models do not fully reflect the clinical pathology of human infections because, in permissive species, RSV generally replicates without overt symptomatology[7]. Taken together, these factors have contributed to the current clinical situation in which the only available therapeutic option is the teratogen ribavirin, while the dramatic failure of early attempts to develop an effective RSV vaccine have restricted prophylactic therapy to that of passive immunization provided by the recently introduced humanized monoclonal antibody Synagis (palivizumab, MEDI493)[4,8,9]. However, interest in developing new clinically

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effective agents for the prevention and treatment of RSV has begun to rise together with a heightened awareness of the prevalence of RSV and its role in disease pathology. This review will provide an overview of recent progress towards the identification and development of inhibitors of RSV against the background of an emerging appreciation of the clinical significance of this respiratory tract pathogen.

Clinical background

In the Northern Hemisphere, RSV typically manifests as annual outbreaks in most communities, emerging in mid-November, peaking in January and February and dissipating by April[10–12], although there is burgeoning evidence that RSV might be a year-round epidemic[13]. Propagation of outbreaks is facilitated by the ease of transmission of RSV, which occurs by exposure to droplets of respiratory secretions of infected individuals[14], and infection rates are high after introduction into families[15]. Almost all children become infected with RSV during their first two years, and symptomatic reinfection is a common occurrence throughout life, owing to an inadequate immune response that is of limited durability[16].

For most individuals, RSV infection is restricted to the upper respiratory tract (URT), producing an influenza-like illness or appearing as a persistent cold that is self-limiting and not usually associated with significant clinical complications. However, in certain segments of the population, RSV infection produces a lower respiratory tract (LRT) disease of considerable severity, in some circumstances leading to significant morbidity and mortality. Infants are particularly vulnerable, with primary infection producing cough, rhinitis and a mild fever over a period of four to five days. In at least 40% of these individuals, the RSV infection progresses to involve the LRT, causing wheezing, severe cough, and increased respiratory rate, symptoms associated with bronchiolitis and/or pneumonia. Peak RSV activity has been found to correlate highly with mortality from LRT illness in children aged 1–11 months, although in older children (24–59 months), influenza was the more important infection[17]. RSV is a particularly serious infection in the immature lungs of children born prematurely[18], or those with compromised cardiopulmonary function owing to an underlying condition, including congenital heart disease[19] and chronic lung disease (bronchopulmonary dysplasia)[20]. Mortality rates in these patient populations were high before the advent of the sophisticated intensive care support available in contemporary hospitals. Not surprisingly, RSV is also documented as problematic in children whose immune function is compromised[21].

As children age, reinfection with RSV is common but generally of lesser severity, although it can exacerbate other pathologies associated with lung function. Most prominent is asthma[22,23], for which there is some evidence that the incidence of disease might be linked to an RSV infection in early life[24], although a recent clinical study suggests that this might be restricted to children under the age of 11 (Ref. 25). Otitis media is a common childhood disease affecting approximately 12 million children per annum in the USA (Ref. 26) for which bacteria are generally considered to be responsible. However, the clinical response of otitis media to antibacterial drugs is often incomplete and has led to an examination of the role of viruses as etiological agents[27,28]. Indeed, in a recent clinical study, RSV was identified as the principal viral etiological agent in acute otitis media[28].

Among immunocompromised adults[29], bone marrow transplant recipients[30] are particularly vulnerable to the effects of RSV. RSV is the leading cause of infection in this severely compromised population and mortality rates can be extremely high (80%) in the absence of ribavirin therapy[31]. RSV has also been reported as a problem in individuals undergoing cardiac[32], renal[33] and lung[34] transplants and in leukemia patients[35]. An appreciation of the importance of RSV as a problematic infection in the adult community is also beginning to emerge[36], particularly in the elderly[37,38], where it is frequently misdiagnosed as influenza[39] and its impact underestimated[38]. Indeed, epidemiological studies have estimated that RSV is responsible for 60–80% greater mortality than influenza during the winter season[39,40].

Virus structure and function

RSV is a non-segmented, negative-stranded RNA virus of the Paramyxoviridae family that has been classified into two subgroups, designated A and B, based on the serological differences in viral envelope proteins[6]. RSV replicates in the cytoplasm of infected host cells and buds through the apical membrane, thereby acquiring its lipid envelope. The entire genetic material is associated with virus-encoded proteins, including the polymerase, which together form the nucleocapsid and are packaged in the virion[6]. The 15,222 nucleotide genome encodes ten major viral proteins (Fig. 1) of which three, the F (fusion), G (attachment) and small hydrophobic SH (unknown function) proteins, are expressed on the virion surface, anchored in the lipid membrane[41]. Two proteins, designated M and M2-1, and three viral RNA-associated proteins identified as N (major nucleocapsid protein), L (viral RNA polymerase) and P (phosphoprotein) are also packaged in

the virion. In addition, two non-structural proteins, NS1 and NS2, accumulate in the cytoplasm of infected cells but are present in mature virions in only trace concentrations. An 11th gene, a 90-residue protein, designated M2-2 is contained as a second open reading frame within the M2 mRNA (Ref. 6).

The RSV F protein

Of the surface proteins, only the F protein has emerged to date as a target for therapeutic intervention, both as the epitope for monoclonal antibodies and vaccines and as a target for small-molecule antiviral agents, perhaps reflective of its crucial role in viral entry and relative lack of genetic heterogeneity[42]. Recombinant viruses devoid of either the SH protein[43] or both the SH and G proteins[44], engineered as candidate vaccines, retain their infectivity *in vitro*, although they are less virulent *in vivo*. In common with the fusion proteins of many viruses, the F protein is synthesized as a single, *N*-glycosylated polyprotein of 574 amino acids, designated F_0, which requires activation to its fusion-competent form, disulfide-linked subunits F_1 and F_2, by proteolytic cleavage at the C-terminus of F_2 (Ref. 6). This processing is accomplished by a host cell endoproteinase with trypsin-like sequence specificity that unmasks the fusion peptide at the N-terminus of F_1, a sequence of 15–17 hydrophobic amino acids, which is homologous to other viral fusion peptide sequences. The processed protein assembles on the surface in a multimeric form, although the exact composition of the oligomeric species (trimeric or tetrameric) remains a topic of some controversy[6]. The F protein is thought to mediate fusion of virus and host cell membranes in a fashion that is common to many viruses, beginning with an initial movement of the F_2 protein, although the trigger for this event remains to be determined. As a consequence, the F_1 protein is destabilized and subsequently undergoes a conformational rearrangement, unmasking the fusion peptide and facilitating its insertion into the host cell membrane.

The RSV G protein

The G protein, which mediates attachment of RSV to host cells, comprises 289–299 amino acids, depending on the strain. However, the molecular mass of the G protein in mature form is 84–90 kDa, considerably larger than predicted, and indicative of extensive post-translational modification[6]. The latter includes not only *N*- and *O*-glycosylation but also palmitoylation, and the mature protein is thought to be trimeric in nature. The RSV G proteins of A and B strains exhibit limited sequence identity (~53%), whereas G proteins from viruses of the same antigenic group can vary by up to 20%. The host cell proteins that engage the RSV G protein, maintaining the F protein in close proximity to host cell membranes and thereby enhancing the chances that the F protein will initiate a fusion event, have yet to be definitively identified.

Other RSV proteins

Of the other viral proteins that have been studied, the small hydrophobic SH protein is a short, integral membrane protein comprising 64 amino acids, with the C-terminus extracellular. However, the precise role of the SH protein in the virus life cycle remains enigmatic and, although it facilitates fusion, it is not an essential component in this process. The RSV polymerase, designated the L protein, is one of three proteins packaged in the nucleocapsid along with the N and P proteins. Whereas viral polymerases have proven to be classic targets for therapeutic intervention, the RSV polymerase is poorly active *in vitro* and the enzyme is not well characterized[45].

Vaccines and monoclonal antibodies for the prevention and treatment of RSV infection

Early studies

The development of an effective vaccine for RSV has been extremely slow, in part because of major problems encountered during early clinical trials. Attempts to develop a safe and effective RSV vaccine over 30 years ago using a formalin-inactivated virus produced an unanticipated outcome.

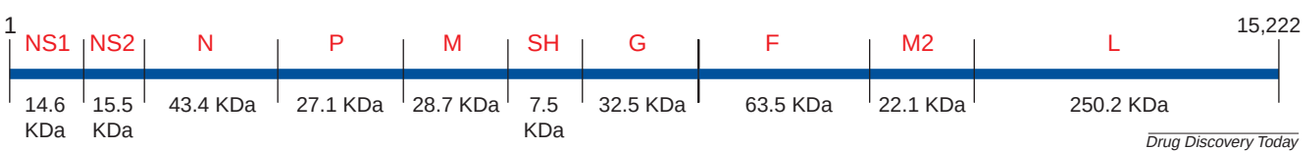


Figure 1. *The respiratory syncytial virus (RSV) genome depicting the location of the ten gene products and the size of the protein encoded by the individual RNA segments.*

Not only were vaccinees not protected against a subsequent wild-type RSV infection, but the severity of LRT infection in these children was actually exaggerated compared with non-vaccinated controls[46,47]. Two of the inoculated infants died and the underlying mechanism for this rather dramatic failure remains to be completely elucidated. However, current theories implicate a predominant T helper cell type 2 (Th2)-like immune response produced by the vaccine that did not adequately prime $CD8^+$ cytotoxic T cells to produce a pattern of $CD4^+$ cells similar to that induced by the natural infection[47]. The objective of RSV vaccines is to induce both a systemic, serum antibody-based immunity to protect against LRT infection and local, secretory-based immunity to reduce URT infection. Subsequent attempts to develop RSV vaccines have focused on live, attenuated viruses, inactivated viruses and subunit elements, with genetically engineered (cDNA-derived) and DNA-based vaccines[48] emerging more recently[47]. However, given the problems encountered earlier, all of these approaches need to be examined in a somewhat cautious fashion.

Attenuated live virus vaccines

Early studies with live virus vaccines were unsuccessful because of difficulties associated with generating candidates that possessed an optimal attenuation profile. However, success has been achieved by combining two strategies of attenuation: cold passaging and temperature sensitivity, which were initially evaluated individually. The most promising of these intranasally administered vaccine candidates developed to date is an RSV strain designated cpts248/404, which contains eight nucleotide changes from the wild-type virus that predict seven alterations at the amino acid (aa) level – one in the N protein, two in F, and four in the L (polymerase) protein[47]. Two related vaccines, cpts530/1009 and cpts248/955 (both developed by National Institutes of Health, Bethesda, MD, USA), have been advanced into clinical trials, where they have been found to possess stable phenotypes but insufficiently attenuated for more advanced study[47].

However, the advent of reverse genetics has made development and fine-tuning of potential vaccine candidates a reality. The ability to create an infectious RSV through a full-length DNA intermediate provides an opportunity for iterative rounds of defined mutations to develop a candidate with the desired attenuation and immunological characteristics[49]. In this fashion, mutations with known attenuation characteristics can be mixed and matched to create the desired effect. For example, although cpts248/404 is not sufficiently attenuated, reverse genetics enables the addition of another mutation, such that the newly produced cpts248/404/1030 virus exhibits enhanced attenuation and temperature sensitivity[47,50].

The same approach can be taken for constructing viruses with defined deletions. For example, recombinant virus with a deletion of either the gene encoding NS2 or SH exhibits normal growth kinetics in tissue culture, but is attenuated in chimpanzees[51]. In the same way, Wertz and coworkers[52] have found that changing the gene order within non-segmented negative strand viruses results in a virus with attenuated growth characteristics in animals. The application of these reverse genetic techniques is leading to a new era in the development of RSV vaccines, offering considerable promise that effective vaccine candidates will be identified in the near future.

Subunit vaccines

The potential of several subunit vaccines derived from both the RSV G glycoprotein and the genetically more homogenous F glycoprotein, as well as chimeric F and G combinations, have been examined preclinically. Two of these, designated PFP-1 and PFP-2, in which the RSV F protein is purified by immunoaffinity and ion exchange chromatography, respectively, and subsequently absorbed on alum, have been advanced into clinical trials[47]. These vaccines were well tolerated in healthy adults, children over the age of 12 with or without the underlying pulmonary problems associated with cystic fibrosis or bronchopulmonary dysplasia, and the elderly. In most vaccinees, moderate (greater than fourfold) increases in RSV-neutralizing antibodies were observed, but some studies suggested the potential for limited efficacy in this regard. As a consequence, these and related RSV F subunit vaccines are being examined in conjunction with adjuvants as a means of further enhancing antibody titers[47,53].

Passive immunization with monoclonal antibodies

Whereas vaccine development has been fraught with difficulty and complexity, the development of antibodies designed to induce passive immunization has proved to be far more successful, culminating with the introduction of Synagis for clinical use in the USA in September 1998 and in Europe in the summer of 1999 (Refs 54,55). Early studies demonstrated the potential of this approach using standard human immunoglobulin, which showed efficacy in clinical trials[55]. However, difficulties associated with producing standard human immunoglobulin with reproducibly adequate RSV neutralization test titers led to the development of a more reliable procedure for isolating RSV antibodies from pooled human plasma[55]. This

immunoglobulin, designated RSVIG, was evaluated in clinical trials based on the demonstration of efficacy in the cotton rat model of RSV infection. In clinical studies conducted in high-risk infants and children, RSVIG, administered via monthly intravenous infusions at doses of 500 or 750 mg kg^{-1}, proved to be a safe and effective prophylactic for the prevention of LRT infection[55,56]. Several additional studies established the efficacy and limitations of RSVIG in a clinical setting[57–60] and it was concluded to be a cost-effective treatment[61]. RSVIG was marketed by MedImmune as RespiGam in 1996 for the prevention of serious LRT infection caused by RSV in children under 24 months of age with underlying cardiac problems or a history of premature birth.

Evolution of immunoglobulin therapy has focused on the development of humanized mouse monoclonal antibodies, two of which have been evaluated in the clinic. RSHZ19 (SB209763; SmithKline Beecham, Philadelphia, PA, USA, licensed from Scotgen Pharmaceuticals, Menlo Park, CA, USA) showed efficacy *in vivo* in mice and cotton rats but, despite acceptable pharmacokinetic properties, failed to protect children born prematurely or with bronchopulmonary dysplasia against RSV infection[62]. As a consequence, development of this antibody has been terminated. By contrast, MEDI493 (palivizumab)[63] has proven to be a more efficacious antibody in humans[64–66] than RSHZ19 (SB209763), possibly because of enhanced potency[67], and MedImmune launched MEDI493 in the USA as Synagis in September 1998. Synagis is approved for use in a broader patient population than RespiGam, being suitable for prophylactic administration to pediatric patients at risk of RSV disease, and might exhibit greater efficacy. More recently, clinical studies have begun to evaluate the potential of Synagis as a therapeutic agent for the treatment of established RSV infections[68] and for use in the immunocompromised population[31]. A co-marketing arrangement with Abbott's Ross Division (Chicago, IL, USA) has helped to propel sales of Synagis, which is administered as a series of monthly intramuscular injections, to over $200 million during the 1998–1999 RSV season.

Small-molecule inhibitors of RSV replication for the treatment and prevention of RSV infection

The successful launch of Synagis has demonstrated an unmet clinical need for an effective RSV prophylactic agent. However, the therapeutic potential for Synagis remains to be established and the cost of a course of treatment is expected to be high. The identification of small, drug-like molecules that effectively, specifically and potently interfere with RSV replication is beginning to attract considerable attention[5,69–71]. Most inhibitors of RSV disclosed in the literature to date have been discovered by a strategy of screening using a tissue cell culture assay, a consequence of the currently limited understanding of molecular aspects of the RSV life cycle that has prevented a more fundamental, mechanism-based approach[5,69–71]. However, this situation has begun to change recently with the application of fusion peptide analysis technology by Trimeris (Durham, NC, USA), which has provided, at some level, a structural basis for the design of drugs that target the fusion process. In addition, the application of antisense approaches to RSV inhibition is under active examination, providing an alternative rationale on which to base antiviral drug discovery.

Nucleoside analogs

The synthesis of nucleoside analogs and their evaluation using tissue culture assays has provided a fundamental and successful platform for antiviral drug discovery for many years. However, although this strategy has produced effective and efficacious drugs for many viral infections, notably absent from the clinical pharmacopoeia are nucleoside analogs that treat viruses replicating only via RNA-based intermediates. Nucleoside analogs that act as precursors to potent and effective RSV polymerase inhibitors in cell culture have yet to be definitively described[69,72].

Ribavirin. This compound (Fig. 2) is the only clinically approved small-molecule therapy for the treatment of RSV infections[4,73,74] but the precise mode of action of this nucleoside analog remains to be established[75]. Ribavirin (marketed for RSV by ICN Pharmaceuticals, Costa Mesa, CA, USA) is effective *in vitro* against a broad spectrum of RNA viruses, with inhibition of influenza virus being the most well studied. Ribavirin is believed to inhibit influenza virus replication at three levels: the monophosphate derivative is an inhibitor of host cell inosine monophosphate dehydrogenase (IMPDH), thereby depleting intracellular pools of GTP and interfering with viral RNA synthesis, whereas the triphosphate has been shown to inhibit both the polymerase and the capping of the 5′ end of viral mRNA (Refs 73,75). Because guanosine can reverse the RSV inhibitory activity of ribavirin in cell culture, inhibition of IMPDH contributes to RSV inhibition[76].

Ribavirin inhibits RSV in cell culture with IC_{50} values in the 8–20 μM range[77] and shows efficacy in the cotton rat model following either topical or systemic administration[78]. Although lower doses reduced viral titers in lung turbinates, an intraperitoneal dose of 200 mpk (milligrams per kilogram) three times-a-day was necessary to achieve a 1.1-log reduction in lung viral titers. The high doses

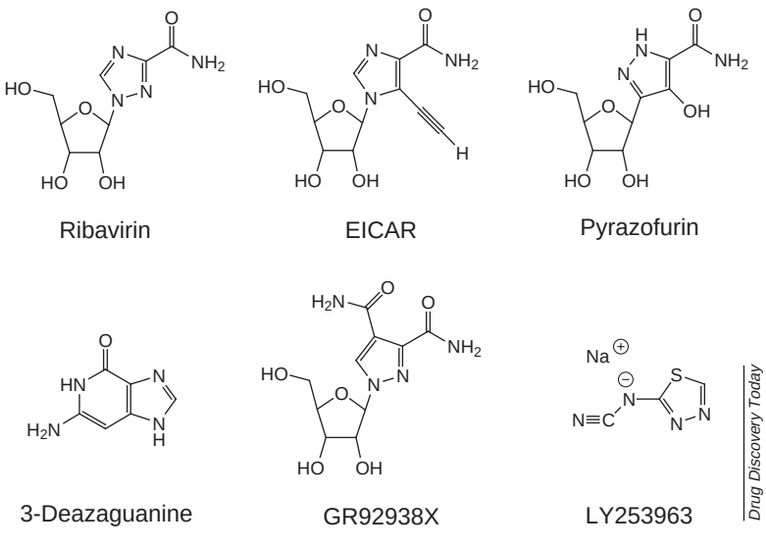


Figure 2. *Nucleoside inhibitors of respiratory syncytial virus (RSV).*

required probably reflect the poor bioavailability at the site of infection because [^{3}H]-ribavirin was found primarily in the liver, with only low levels of radioactivity associated with lung tissue, although calculations suggested adequate drug concentration in the lung[78]. Aerosol delivery of ribavirin initiated 1 h after virus inoculation provides a much more effective means of controlling RSV replication in cotton rats and this mode of drug administration is currently preferred in the clinic. To observe a robust antiviral effect in cotton rats, a concentration of 2–4 mg ml^{-1} of ribavirin in the aerosol reservoir was necessary (1 mg ml^{-1} was less effective), with calculations suggesting that this drug concentration translated into an effective dose of 5 mpk day^{-1}.

Other IMPDH inhibitors. Several analogs of ribavirin have been found to inhibit RSV replication *in vitro* but none have been evaluated in animal models of RSV infection. The broad-spectrum antiviral EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide, Hokkaido University, Sapporo, Japan and Rega Institute for Medical Research, Leuven, Belgium; Fig. 2) is 30-fold more potent than ribavirin in cell culture assays, where inhibition is reversed by guanosine, implicating inhibition of IMPDH as a contributing event[76,79]. The monophosphate metabolite of EICAR has been shown to be an irreversible inhibitor of IMPDH (Ref. 80). Pyrazofurin (Fig. 2) is even more potent, with an average EC_{50} of 70 ng ml^{-1} across a series of clinical and laboratory strains of RSV replicating in HeLa cells[81]. However, the biochemical pharmacology of pyrazofurin differs qualitatively from ribavirin in that the latter primarily inhibits syncytium formation, whereas the former also effectively inhibits viral antigen synthesis[81]. The biochemical pharmacological profile of 3-deazaguanine (Fig. 2) is comparable to that of pyrazofurin, although it is a markedly less potent antiviral agent[81].

Although equipotent with ribavirin as an inhibitor of RSV in HEp-2 cells, the pyrazole-based nucleoside analog, GR92938X (Glaxo-Wellcome, Greenford, Middlesex, UK; Fig. 2), is considerably more specific, being inactive against influenza A and B strains as well as parainfluenza 2 virus[82]. Consistent with this profile and in contrast to ribavirin, there was no evidence from a whole-cell evaluation that GR92938X interfered with IMPDH. LY253963 (Eli Lilly, Indianapolis, NJ, USA; Fig. 2), the prodrug of an inhibitor of IMPDH that requires sequential glycosylation and phosphorylation to express its biological activity, demonstrates RSV inhibitory activity *in vitro* and *in vivo*[83]. In cotton rats, intraperitoneal administration of LY253963 at doses of 3 mpk day^{-1} resulted in a significant reduction in pulmonary viral titers, but the compound showed no demonstrable activity when administered orally.

Inhibitors of virus adsorption and entry

Several agents have been characterized as inhibitors of RSV adsorption, although a detailed understanding of their mode of action remains to be elucidated[69]. Prominent among this class of inhibitor are the polyoxometalates[84], complexes of cationic alkaline earth metals coordinated with the negatively charged oxygen atoms of cryptate-like structures that, as a structural class, have been found to inhibit a variety of viruses in cell culture[85]. Several classes of polyoxometalate were found to inhibit cytopathy induced by both laboratory and clinical strains of RSV and bovine RSV (BRSV), with the most effective compounds expressing submicromolar IC_{50} values in the absence of overt cytotoxicity.

Peptidic fusion inhibitors. The discovery that peptides based on specific sequences of the HIV-1 glycoprotein gp41 fusion peptide interfered with virus fusion and prevented infectivity in cell culture[86] has provided an opportunity to examine more deeply aspects of the virus–host cell fusion process[87,88]. Using a computer-based searching strategy designated CAST (computerized antiviral searching technology), the fusion peptides from a series of paramyxoviruses were examined to identify domains of heptad repeats that are hypothesized to interact with each

other during later stages of the fusion process. For the RSV F protein, three such domains were identified (Fig. 3), two in F_1 and a unique sequence, designated HR3, in the F_2 protein[88]. Synthetic peptides comprising 35 amino acids that scanned the HR2 domain in an overlapping fashion demonstrated more potent RSV inhibition than analogous peptides designed from the HR1 domain, whereas those based on HR3 were inactive. T118 (Fig. 4) was the most potent RSV inhibitor to emerge from this analysis and exhibited an IC_{50} of 51 nM in a cell culture assay in HEp-2 cells[88]. The precise mode of action of T118 at the molecular level remains to be determined, but in an analogy to DP178, an inhibitor of HIV-1 fusion[87], T118 is postulated to function by interfering with an intramolecular interaction between the HR1 and HR2 domains, which develops during the later stages of the fusion process. This occurs after the conformational rearrangement of the F protein and is a crucial event in the fusion pathway[88]. Validation of this antiviral strategy in a clinical setting has recently been established in the context of HIV-1-infected individuals whose plasma viral RNA declined by almost 2-log in response to twice daily intravenous doses of T20 as monotherapy[89].

Trimeris has recently disclosed a series of small-molecule inhibitors of RSV infectivity in cell culture that are based on a benzanthrone skeleton[90]. The representative structure depicted in Fig. 4 is a potent inhibitor of RSV infection, IC_{50} = 0.04 μg ml^{-1}, which displays a CC_{50} (concentration of drug that causes death to 50% of the cells) of 4.15 μg ml^{-1}, a selectivity index that appears to be typical of this structural class. Although the results of *in vivo* experiments are not disclosed, this chemotype represents a new and interesting inhibitor of RSV that might function by inhibition of fusion (M.R. Johnson *et al. 36th Interscience Conference on Antimicrobial Agents and Chemotherapy*, 15–18 September 1996, New Orleans, LA, USA, abstract S114).

CL387626. This compound (Fig. 4) is the most thoroughly characterized of a series of symmetrical triazine derivatives that were discovered by HTS methods using a cell-based assay[91,92]. CL387626 (Wyerth-Ayerst Research, Pearl River, NY, USA) inhibits a variety of laboratory and clinical isolates of RSV A and B strains replicating in HEp2 cells with IC_{50} values of ~0.05 μM, but is inactive against measles, parainfluenza virus-3 and adenovirus-5 infections, thereby demonstrating excellent virus specificity. Structure–activity studies with CL387626 have revealed that the RSV inhibitory activity is sensitive to even relatively small changes in both the core of the molecule and the peripheral structural elements. Insights into the mode of action of CL387626 have been developed using several techniques that implicate reduction of the multimeric state of the RSV F protein contributing to inhibition of the fusion of host cell and RSV membranes.

CL387626 is an effective antiviral agent in the cotton rat model of RSV infection, where the compound demonstrates an intriguing pharmacological profile. Antiviral activity *in vivo* is seen only when CL387626 is administered intranasally at least 1 h before virus inoculation, with no demonstrable therapeutic efficacy observed. A dose of 30 mpk of CL387626 given intranasally reduced viral titers in lung samples by over 3-log g^{-1} of lung tissue, although a robust dose–response relationship could not be established[91]. Remarkably, the compound provides protection against RSV infection when a single prophylactic dose is administered as much as five days before virus inoculation; however, efficacy appeared to be better when drug and virus administration were temporally closer. Not surprisingly, considering the molecular size and charge associated with CL387626, no antiviral activity was seen after intraperitoneal drug administration. Preclinical development of CL387626 is not currently being actively pursued, in

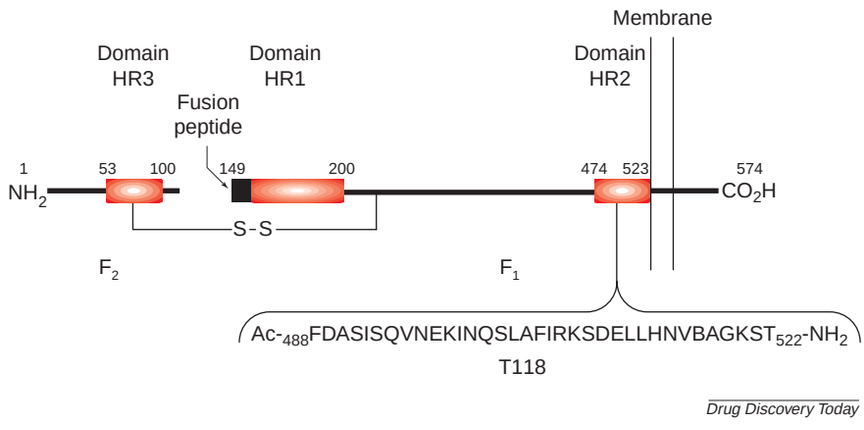


Figure 3. *The respiratory syncytial virus (RSV) F protein depicted in its functional, two-chain form, identifying the location of the fusion peptide, cleavage site, membrane-spanning domain and the origin of F protein sequences that have been found to inhibit RSV–host cell fusion.*

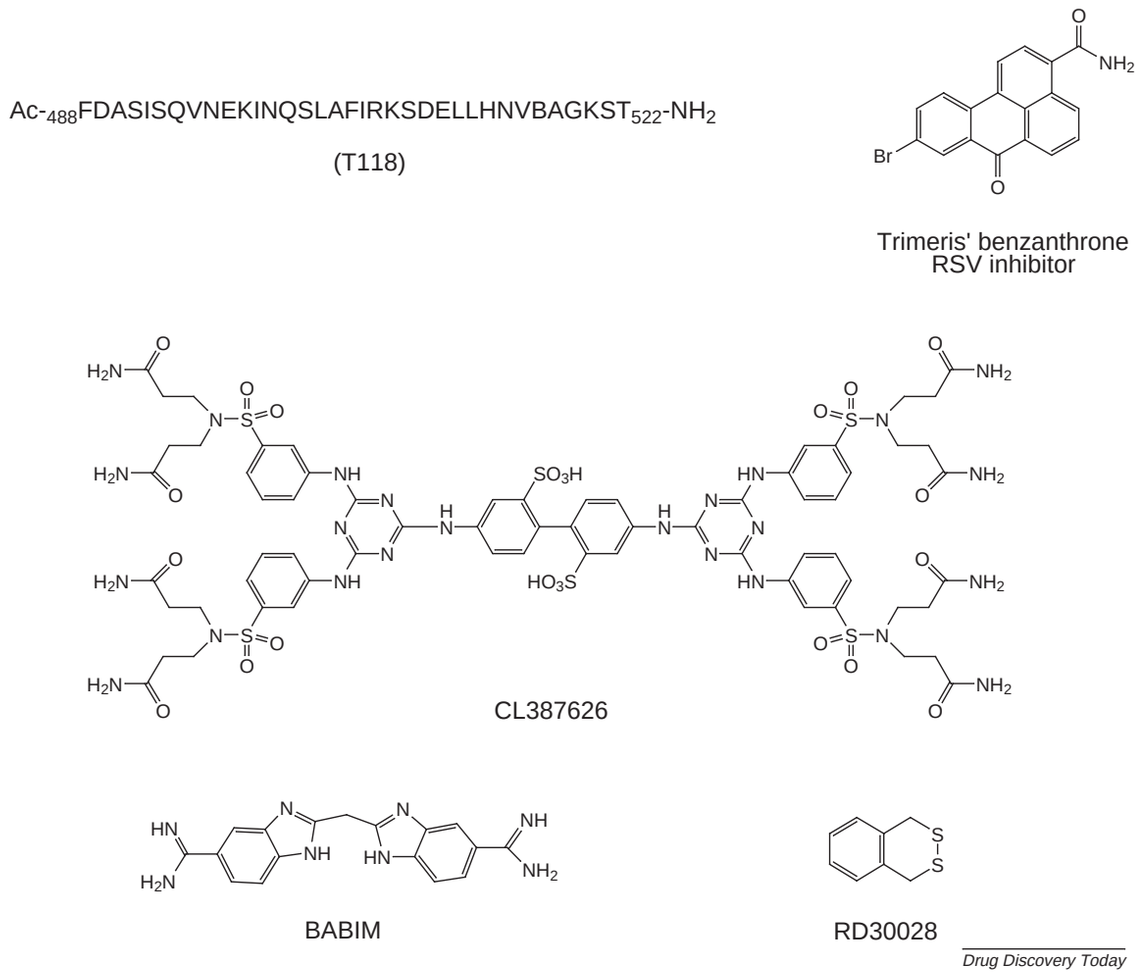


Figure 4. *Inhibitors of respiratory syncytial virus (RSV) fusion with host cells.*

favor of alternative members of the series that might offer significant clinical advantages[91].

BABIM. Another compound characterized as an RSV fusion inhibitor is BABIM [bis-(5-amidino-2-benzimidazolyl)methane, University of North Carolina School of Medicine, Chapel Hill, NC, USA; Fig. 4], which was originally synthesized as a member of a series of heterocyclic amidine derivatives designed to probe structure–activity relationships of broad-spectrum, trypsin-like protease inhibitors[93]. This compound was subsequently found to possess potent RSV inhibitory activity in cell culture assays, where several lines of evidence suggested interference with the fusion process[94]. BABIM has shown activity in the cotton rat model, where a daily dose of 25 mpk intraperitoneally for four days resulted in >10-times reduction in viral titers recovered from the lungs; in animals subject to cyclophosphamide-induced immunosuppression, a seven day treatment protocol produced more than a 100 times reduction in viral titers[95]. However, *in vitro* studies[94] have not been able to effectively discriminate RSV inhibition from protease inhibition, which, coupled with the discovery that BABIM is a potent inhibitor of the respiratory tract protease human mast cell tryptase[96], provides for a situation of even greater complexity *in vivo.*

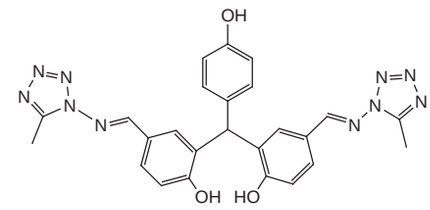
Other small-molecule inhibitors of RSV

The RSV inhibitory properties of the structurally simple fused bicyclic disulfide RD30028 (Rational Drug Design Laboratories, Fukushima, Japan; Fig. 4) have been examined in some detail, including evaluation in an *in vivo* model of RSV infection in mice[97,98]. RD30028 inhibits laboratory and clinical isolates of RSV A and B strains in cell

culture with IC_{50} values of ≈4 μM and a CC_{50} of 271 μM, but is inactive against a panel of viruses that included influenza A, measles, HSV-1, HSV-2 and human cytomegalovirus (HCMV)[92]. Although the disulfide element of RD30028 is inherently chemically reactive, virus incubated with the compound for 1 h remains infectious, inconsistent with an irreversible modification of viral proteins. RD30028 appears to act at a stage late in the virus replication cycle because inhibition in cell culture is maintained when the drug is administered up to 8–10 h after virus inoculation.

Further insight into the mode of action was gleaned by isolating resistant viruses, obtained by serial passage in the presence of inhibitor, and sequencing the mutants. Resistance was mapped to the F protein with residue 276 (in F_1) found to be altered from Asn to Tyr in one strain, whereas alteration of a Lys to an Asp at position 80 of the F protein, in the HR3 domain of F_2 identified by Trimeris, conferred resistance in a second strain. It was concluded that RD30028 interferes with the synthesis or intracellular processing of the RSV F protein, leading to the production of defective virus particles[99]. In immunosuppressed mice infected with RSV, aerosol administration of RD30028 for 2 h twice daily for three days beginning 24 h after virus inoculation produced an antiviral effect that was dependent on the dose. Drug concentrations of less than 1 μg ml^{-1} in the aerosol reservoir were poorly efficacious; however, reservoir concentrations of 1.25, 2.5 and 7 μg ml^{-1} of RD30028 resulted in 50%, 64% and 64% reduction in pulmonary viral titers, respectively[98]. Ribavirin, used as a positive control, produced a 60% reduction in viral titers at a reservoir concentration of 60 mg ml^{-1}. Histopathological analysis of lung tissue of drug-free, infected animals revealed evidence of interstitial pneumonia, whereas the lungs of treated animals were similar to those of virus-free controls.

A recent patent application by Viropharma (Malvern, PA, USA) discloses a series of potent inhibitors of RSV replication in cell culture[100]. The representative example depicted in Fig. 5 inhibits RSV A and B strains and a panel of 34 clinical isolates in cell culture, with IC_{50}s ranging from a few hundred pM to 80 nM. This compound (VP14637) is relatively selective for RSV being inactive against parainfluenza 3 virus, mumps, measles, influenza A and herpes simplex virus 2 in cell culture at a concentration of 3 μM, a limit imposed by the poor solubility of the compound (D.C. Pevear *et al. 13th International Conference on Antiviral Research*, 16–21 April 2000, Baltimore, MD, USA, Abstract 57). Time-of-addition studies using a single cycle of viral replication indicate that VP14637 acts at an early stage in the virus life cycle and resistant viruses have mutations in the F gene, suggesting inhibition of fusion as the mode of action. This is consistent with the broad-spectrum inhibition against several RSV strains and clinical isolates. Viropharma has licensed Battelle Pulmonary Therapeutics' inhalation drug delivery device to deliver VP14637 topically and are proceeding to evaluate the compound in the cotton rat model of RSV infection as a prelude to initiating clinical studies.



RSV inhibitor chemotype (Viropharma)

5'-AAAAATGGGGCAAATAAATC-3'

(V590)

sp(5'A2'p)3A-Bu2-5'-ATGGTTATTTGGGTTGTT-3'-3'T5'

(2'-5'A-8281-8299)

sp(5'A2'p)3A-Bu2-5'-AsAsAsAATGGGGCAAAsTsAsA

(NIH351)

Drug Discovery Today

Figure 5. *Viropharma's (Malvern, PA, USA) small-molecule inhibitor of respiratory syncytial virus (RSV) and antisense nucleotide inhibitors of RSV.*

Antisense oligonucleotides

Because of the difficulties frequently associated with identifying inhibitors of viral proteins and, in many cases, a limited range of targets, viruses have emerged as attractive candidates for the development of therapeutics based on antisense oligonucleotides[101]. This technology has been focused on RSV with the principle recently validated under cell culture conditions. V590 (Hybridon, Worcester, MA, USA; Fig. 5), a 20-residue phosphorothioate oligodeoxyribonucleotide targeted to the junction of the RSV NS1/NS2 genes, inhibited RSV replication in HEp-2 cells with an IC_{50} of 0.64 μM and an IC_{99} of 6.6 μM (Ref. 102). Because V590 contains a GGGG motif that can be associated with antisense-independent activity, a scrambled version of V590 that maintains the GGGG element was examined as a control. Although this oligonucleotide

was only four-times weaker, a closer analysis of the effects of V590 revealed specific cleavage of RSV genomic RNA at the targeted sequence, suggesting that an antisense mechanism contributed to the antiviral activity[102].

An enhancement of the antisense-based approach that has proved to be more successful in an *in vitro* setting has been the incorporation of a 2′-5′A oligonucleotide at the 5′ terminus of the synthetic DNA (Refs 103,104). This structural modification provides for a more selective and enhanced rate of degradation of the target mRNA by activation of a 2′-5′A-dependent ribonuclease (RNase), an enzyme discovered as a consequence of its induction by interferon. Using MFOLD (Muchael Zuker, Washington University, St Louis, MO, USA), a computer program that analyses the secondary structure of RNA, four sites in the M2 and three sites in the L (polymerase) genomic RNA, one of which was common to both, were identified as suitable targets. Antisense oligonucleotides targeting the M2-encoding RNA displayed greater efficacy in cell culture assays than those directed towards the L gene. The 19 mer 2′-5′A-8281–8299 (Fig. 5), which was designed to hybridize to nucleotides 8281–8299 in a loop structure of the M2 sequence of the RSV genome, was selected for a more detailed evaluation[105,106]. This oligonucleotide, modified at the 5′ and 3′ termini to increase stability, was able to reduce viral yields by 55–70% and 88% at concentrations of 3.3 and 9.9 μM, respectively, under cell culture conditions. Antiviral activity was evident when 2′-5′A-8281–8299 was administered either 4 h before or 2 h after virus inoculation, with the optimal dosing regimen determined to be at 2 and 12 h post-infection. In support of an antisense mechanism dependent on the 2′-5′A element, both a scrambled oligonucleotide and one lacking the 2′-5′A element exhibited greatly reduced antiviral properties, and reverse transcriptase-coupled PCR (RT-PCR) indicated a reduction in RSV M2 RNA in treated cells[107].

Further optimization of this approach was accomplished by a combination of target modification and structural refinement[107]. By targeting the same nine nucleotide RSV gene-start signal consensus sequence as V590, and judiciously incorporating phosphothiorate linker elements designed to reduce non-specific inhibitory properties, NIH351 (Fig. 5) emerged as a potent and selective RSV inhibitor under cell culture conditions. In this study, the 2′-5′A element was again shown to enhance potency, with a 33-fold advantage over the non-2′-5′A-dependent antisense oligonucleotides. Using an A2 virus strain replicating in either the HEp-2 human epidermoid carcinoma cell line or embryonic African green monkey kidney cells, designated MA-104, a single application of NIH351 before virus adsorption reduced virus yield by 50% at 0.3 μM and 90% at 1.0 μM. Under the same experimental protocol, ribavirin was ≈100-times less potent, with an EC_{50} of 30 μM and an EC_{90} of 90 μM. NIH351 showed similar potency against a range of A and B strains of RSV and the related BRSV, although cytotoxicity was observed under some conditions at concentrations as low as 2 μM (Ref. 107). The potential for NIH351 to demonstrate efficacy in animal models of RSV infection has not been disclosed, but the compound is reportedly under development by Atlantic Pharmaceuticals (Raleigh, NC, USA).

Conclusion

An increasing appreciation of the prevalence of RSV and its role in human disease and pathology has led to a burgeoning interest in this virus as a therapeutic target. The success of Synagis as a prophylactic and the significant deficiencies associated with ribavirin as a therapeutic agent are further clarifying RSV as a drug discovery opportunity. However, it is also clear that there are significant hurdles to be surmounted, particularly with small-molecule inhibitors, where potent and specific, orally active antiviral agents have yet to be definitively characterized. Such agents, if brought to market, might also need to be co-administered with an anti-inflammatory agent to alleviate symptoms more effectively[71].

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